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Vaccine xxx (2004) xxx–xxx

Vaccine

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In utero DNA immunization
Immunity over tolerance in fetal life

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Received 5 August 2004; received in revised form 26 October 2004; accepted 1 November 2004

Abstract

The function and plasticity of the developing immune system during embryonic life has been central to immunological thinking for half a century. A classical view is that antigen encountered during fetal life induces a state of acquired immunological tolerance. However, the ability to develop T cell immune responses during the perinatal period would be of great importance against intracellular pathogens. Recent experiments have challenged this notion and shown that neonatal tolerance can be circumvented by extrinsic immunological manipulations. Here, we used DNA immunization targeted at B lymphocytes to induce a CD4 T cell response that could be measured 2 weeks after birth. We conclude that T cell immunity can be programmed in utero by manipulating the parameters of the immune response in the fetal environment. Furthermore, our data suggest that under appropriate conditions the fetal immune system can be programmed to immunity.

Keywords: Fetal B lymphocytes; In utero immunization; Neonatal immunity

1. Introduction

Fifty years ago Medawar and colleagues established that mammals exposed to foreign homologous tissue cells during fetal life never react immunologically, or react to a limited degree only [1]. The principle of actively acquired tolerance during fetal development was thus introduced to become a cornerstone of modern immunology. Exceptions to the rule were soon found with respect to antibody responses to antigenic stimulus [2–7] or manipulation of the idiotypic network during fetal life [8,9], proving the existence of a window of opportunity for actively acquired immunity. In line with Medawar's principle, T cell responses in the perinatal period are also either absent [10–12] or type 2 (Th2) responses (IL-4, IL-5 and IL-10) that are ineffective against intracellular pathogens. Similarly, conventional vaccines against these pathogens administered in the neonatal period are ineffective or induce Th2 responses only [13]. Yet an efficient way to vaccinate infants against intracellular pathogens is a public health priority.

Antigen recognition by T lymphocytes requires presentation of antigen by molecules of the major histocompatibility complex (MHC) [14] at the surface of antigen presenting cells (APC). Recently, several groups [15,16] demonstrated that newborn mice are able to respond to antigenic stimulation provided that antigen is given in the appropriate dose and on the appropriate APC, e.g., bone marrow-derived dendritic cells (DC). The inference from these studies has been that Medawar's experiments were flawed by a defect in DC [17]. Of interest, one of these studies [16] also showed that the induction of cytotoxic T cells (CTL) and type 1 (IL-2 and IFN-γ) cytokines was possible by inoculating a very low dose of infectious virus. Collectively, these studies suggested that

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0264-410X/$ – see front matter © 2004 Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2004.11.046

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neonatal tolerance results from a combination of the same factors that regulate the immune response in the adult organism: the antigen and its presentation to T cells by specialized cells. However, these experiments did not address the essence of Medawar's acquired fetal tolerance as they were performed a few days after birth, not during fetal life. Since, fetal and postnatal life represents different stages in organogenesis and lymphopoiesis, with tissue remodeling being the blue-print of the developmental process, it is still unclear whether the considerations made for neonatal tolerance also apply to the fetal immune system and the principle of actively acquired fetal tolerance. Furthermore, in perinatal infection the immune system has to be ready to react against pathogens already at birth, then the vaccination process should start prenatally.

Indirect evidence from clinical studies has shown that adaptive T cell responses against the malaria parasite [18], cytomegalovirus [19] and respiratory syncytial virus [20] may result from priming in utero pointing to the possibility that the machinery required for these responses is already in place during fetal life. In line with this prediction, a recent study demonstrated that oral DNA immunization of fetal lambs results in the induction of local and systemic T cell immunity [21] but the immunological mechanisms involved were not analyzed. In spite of these reports it is not clear yet what the inner behavior of the fetal immune system is and, if Medawar's actively acquired fetal tolerance does not apply, which are the immunological forces that drive immunity and not tolerance in fetal life.

The experiments reported here were initiated to test the hypothesis that T cell responses can be programmed at the level of the fetal immune system provided that two conditions are met: synthesis of low amounts of antigen in utero and involvement of an APC present and functional in the fetus. To meet these two conditions we adapted to the fetal organism a model of genetic immune programming developed and validated in adult mice, somatic transgene immunization [22]. This consists in a simple injection of plasmid DNA directly into a secondary lymphoid organ. In this model, the plasmid comprises a gene of an immunoglobulin (Ig) heavy (H) chain controlled by a B cell specific promoter. Upon injection, the transgene is internalized and expressed in B lymphocytes which, in turn, set in motion T cell priming [23]. We also found that in the adult mouse T cell priming in vivo occurs in the absence of functional dendritic cells [24], suggesting that B lymphocytes may serve as the first APC. Here we show that the introduction of plasmid DNA in fetuses induces in the majority of instances the expansion of CD4 T cells specific for a MHC Class II restricted peptide expressed in the Ig transgene by spleen lymphocytes of newborn mice, and that these T cell responses are of the Th1 type. These studies hypothesize that fetal liver, the initial lymphopoietic organ, could provide the environment for antigen sensitization and the emergence of antigen-specific T cell immunity soon after birth. The results point to the potential role of fetal B lymphocytes in antigen synthesis and presentation during fetal life or at the transition between fetal and postnatal life.

2. Results

2.1. T cell immunity following DNA priming in utero

Balb/c fetuses were immunized in utero 16 days post coitum by a single injection of plasmid DNA in the peritoneal cavity. To impart antigen specificity, the variable (V) region of IgH transgene was engineered to code for the sequence NANP NVDP NANP (referred hereafter as -NVDP-) in the second complementarity-determining region (CDR). The NVDP 12 mer is a dominant, MHC Class II-restricted Th cell determinant from the malaria parasite *Plasmodium falciparum* [23]. Plasmid $\gamma 1NV^2NA^3$ also codes for $NANP_3$ a malaria B cell epitope, in CDR3 [25]. On average, delivery occurred in 72% of the operated pregnant mothers. Missed deliveries resulted from abortion and premature delivery of fetuses (87% of cases), or reabsorption of fetuses (13% of cases). Only 60% out of a total of 287 injected fetuses were ultimately born. Pregnant mothers carrying 2–5 fetuses at the time of surgery had the highest birth rate, suggesting a negative effect by extensive manipulation of the uterus.

Fetal injection of plasmid $\gamma 1NV^2NA^3$ lead to a CD4 T cell response detectable 2 weeks after birth (Fig. 1A) against -NVDP- in 9 out of 15 litters or 23 out of 43 pups (Table 1). The T cell response, expressed as stimulation index, ranged from 12 to 100 depending on the litter. Interestingly, almost all the pups in responder litters (87%) responded individually. Failure to detect a T cell response in the remaining litters

Table 1
T cell proliferative responses following immunization in utero

	No. of litters	Responder		Percent	SI (range)
		Litters	Pups		
Primary response 14 days after birth					
Plasmid DNA	15	9/15	20/43 (*20/23*)	47 (*87*)	12–100
Control DNA	6	0/6	0/15	0	0.7–1.6
PBS	3	0/3	0/8	0	0.5–1.5
Memory response 4 days after booster					
Plasmid DNA	6	3/6	8/15 (*8/8*)	53 (*100*)	15–74
Control DNA	6	0/6	0/10	0	0.6–1.6

Values in italic refer to responder litters only. Booster immunizations were given on day 45 after birth.

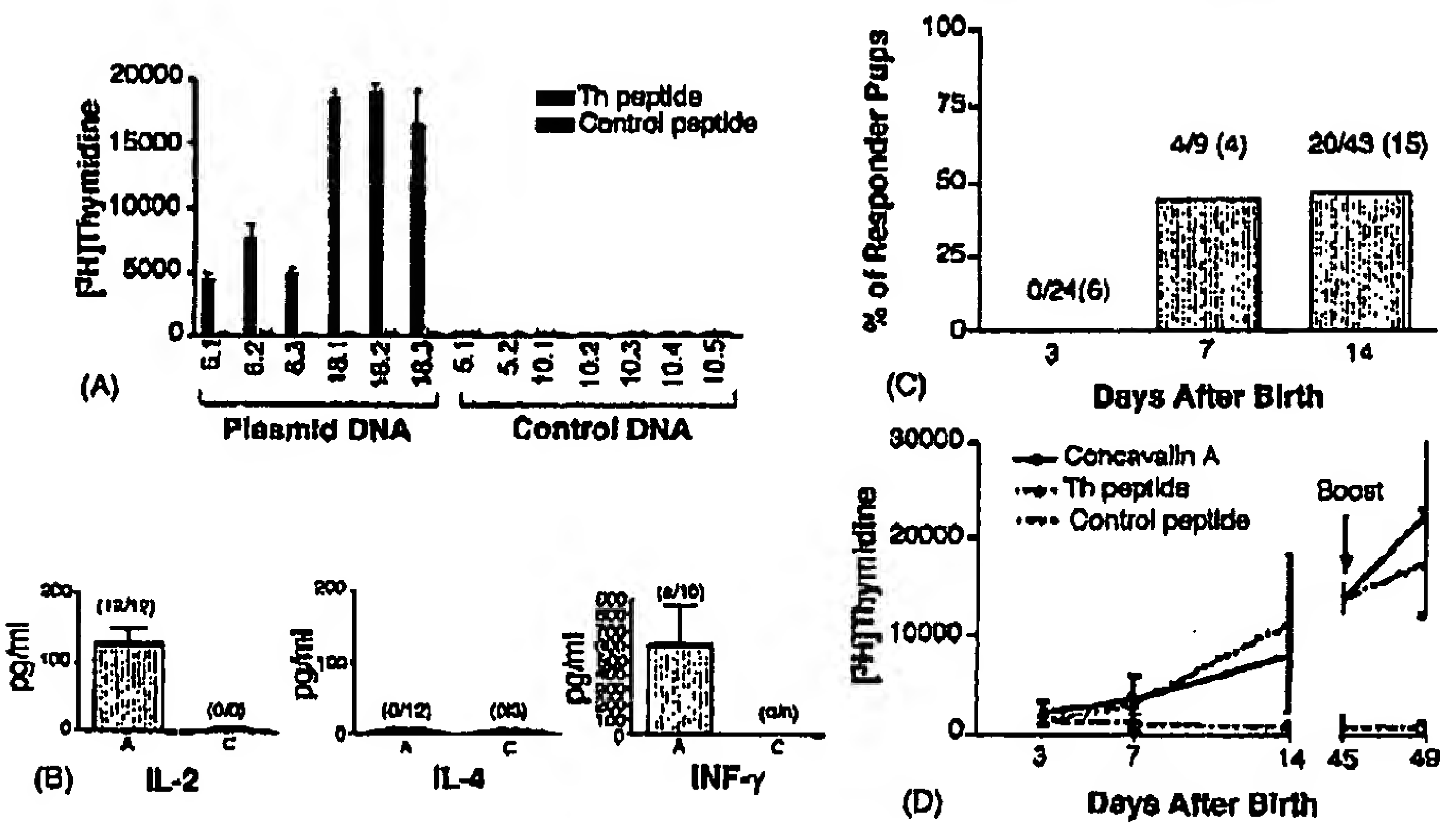

Fig. 1. Antigen-specific activation of T lymphocytes by immunization in utero. (A) Proliferative response of spleen cells from 2-week-old Balb/c mice immunized in utero 16 day post coitum. Two litters were immunized with plasmid DNA $\gamma 1NV^2NA^3$ (six pups) and two litters with plasmid control pSVneo (seven pups). Splenocytes were cultured in presence of the synthetic peptide corresponding to the Th cell determinant -NVDP- or a peptide $(NANP)_3$ as control. Results are expressed as counts per minute (cpm). Numbers along the abscissa identify single mice. Tests were done in triplicate. (B) Detection of cytokines in culture supernatants from responder (R) or control (C) mice. The supernatants were harvested 40 h after culture with synthetic peptide. In parenthesis is the number of samples in which cytokines have been detected over the total number of samples analyzed. (C, D) Kinetics of the proliferative response in the spleen at 3, 7 or 14 days after birth, and 4 days after booster immunization. (C) Percentage of responder pups immunized in utero at each time point. Numbers refer to responder pups over the total analyzed and numbers in parenthesis refer to the number of litters analyzed. (D) Proliferation responses of mice described in panel A. Spleen cells were put in culture with either peptide -NVDP-, control peptide $(NANP)_3$ or ConA. Results are expressed as cpm. Data represent averages of all responder mice. Tests were done in triplicate.

may be attributable to factors known to affect fetal development such as surgery-induced stress [26], variations in the hormonal balance [27], variations in the survival of the plasmid in the fetus (DNA degradation), errors in the evaluation of the gestational day and variations in the diffusion of the DNA after injection. No response was documented in the six litters injected with the pSVneo plasmid control or in the three litters injected with PBS (Table 1). Activated CD4 T cells produced IL-2 and IFN-γ (Fig. 1B) suggesting a prevalent Th1 response. The characteristics of antigen-specific T cell proliferation following in utero immunization are comparable to those induced in adult mice following intra-spleen injection of plasmid DNA [23].

In case of perinatal infection, the immune response has to be active at birth. It became then important to determine the earliest time after birth at which it was possible to detect a CD4 T cell response. Fig. 1C shows that none of the 24 mice tested on day 3 after birth had detectable T cell proliferation. To ensure that lack of T cell priming was not due to a defect in the APC in the 3-day spleen cell cultures, experiments were also performed using as APC lipopolysacharide (LPS) blasts from adult Balb/c mice. Again, no proliferation was observed 3 days after birth suggesting that the defect resides in the T cells and not in the APC. This is in line with the known refractoriness of T cells to polyclonal activation with ConA at this stage of ontogeny (Fig. 1D) [28]. In contrast, 7 days after birth a response was detected in four out of nine pups (two responder litters) suggesting that T cell immunity is already operative 1 week after birth even though the absolute cpm (~2500) were much lower than on day 14. The CD4 T cell response on day 7 was nearly 100% of the proliferative potential measured in response to ConA stimulation. Furthermore, to see if in utero priming established immunological memory, 45-day-old mice immunized as fetuses were boosted with a transfectoma antibody utilizing the same H chain as the one coded by the transgene used for immunization in utero. Table 1 shows a memory response in half of the litters or in 8 out of 15 mice. If one considers only responder litters, a memory response was detected in all mice (8/8). Since, no response was seen in mice immunized with pSVneo and boosted with the transfectoma antibody, we conclude that this is a specific memory response (Fig. 2A). Interestingly, the characteristic Th1 response observed on day 14 persisted in the memory response 4 days after booster (Fig. 2B).

2.2. Role of fetal liver B cells in DNA uptake

To enable a T cell response in vivo following DNA immunization, at least two prerequisites need to be met. One is internalization of DNA by the cell followed by gene transcription and expression. The other is processing and presentation of the transgenic product by the APC to naïve T lymphocytes

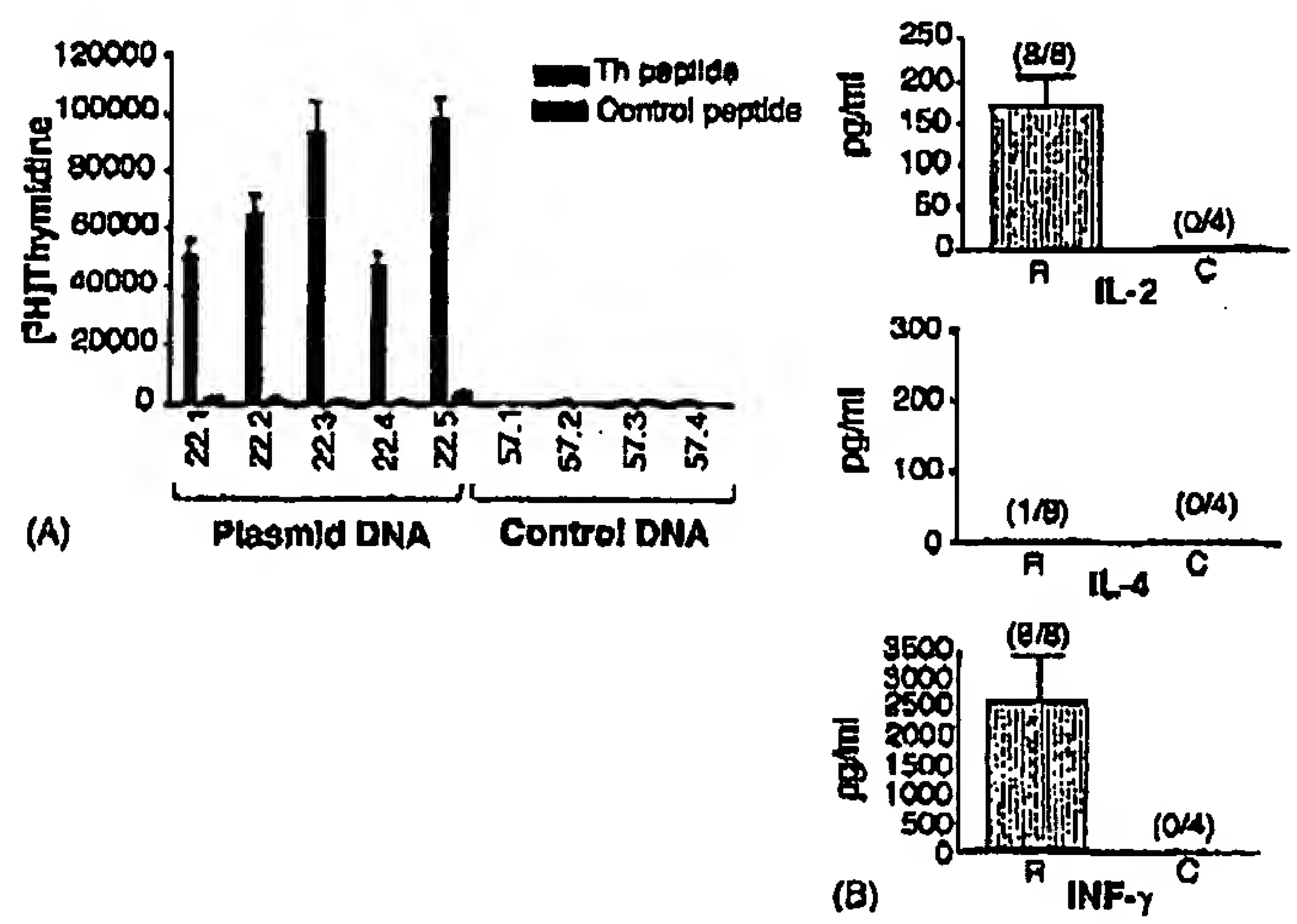

Fig. 2. Immunological memory following in utero priming. (A) Proliferative response of spleen cells in mice primed in utero and boosted with 50 μg of transfectoma antibody γ1NV²NA³ in IFA subcutaneously. Mice were sacrificed 4 days after booster. Splenocytes were cultured with synthetic peptide -NVDP- or $(NANP)_3$ as a control. Results are expressed as cpm. Numbers along the abscissa identify single mice. Tests were done in triplicate. (B). Detection of cytokines in culture supernatants from responder (R) or control (C) mice. The supernatants were harvested 40 h after culture with synthetic peptide. In parenthesis is the number samples in which cytokines have been detected over the total number of samples analyzed.

of appropriate antigen specificity. It then became necessary to document where DNA uptake, transcription and expression might occur and which type of cell may be involved. Initially, we used an IgH chain transgene tagged with EGFP in an attempt to track and visualize green fluorescent cells by fluorescent deconvolution microscopy 24 h after DNA inoculation in fetuses. Examination of total body sections of 17-day-old fetuses failed to provide information as to the localization of green fluorescent cells in the liver or other organs. We concluded that transgenic cells in vivo may be very rare or the expression of transgene very low. Therefore, we decided to take an alternative approach based on the following reasoning. Gaensler and co-workers [29], using an adenoviral vector injected intra-peritoneally as in the present study, successfully demonstrated expression of the transgene in the liver and other organs implying permeability of the fetal peritoneal membrane and diffusion of the viral DNA from the site of injection to the liver (trans-migration) and other organs (through recirculation). Based on this notion we verified by PCR that plasmid DNA also diffuses to the adjacent liver (data not shown). On day 16 of gestation (the time of injection in utero), the fetal liver is the major site of B lymphopoiesis [30–32]. Therefore, since experiments in vivo in adult mice proved that upon intra-spleen injection the DNA is taken up by B lymphocytes [33] and B lymphocytes undergo spontaneous transgenesis in vitro upon contact with DNA [34,35], we decided to directly study the involvement of fetal liver B lineage cells using an ex vivo approach.

B220⁺ cells account for approximately 3–5% of total Ficoll separated fetal liver cells whereas CD19⁺ cells account for approximately 2% [36]. These cells express MHC Class I molecules but only a fraction of them expresses MHC Class II molecules [36,37]. The occurrence of transgenesis was assessed in vitro experiments using the protocol for the spontaneous transgenesis of mature B lymphocytes [34,35] adapted to fetal liver cells. Briefly, Ficoll-separated fetal liver cells were incubated with the EGFP-plasmid together with a plasmid coding for a membrane bound molecule (murine H-2K^k). Co-transfected cells could then be enriched using magnetically labeled anti-H-2K^k antibodies. The transfection was performed in the absence of any carrier molecule or facilitating agent. After enrichment, a small but reproducible proportion of cells (~8% of the gated cells) displayed green fluorescence (Fig. 3A, solid line). These cells were not seen in control liver cells incubated with the plasmid coding for H-2K^k only (Fig. 3A dotted line).

To ensure that the EGFP positive cells were truly committed B lineage cells, we sought expression of Pax-5 and the transgene by RT-PCR. Pax-5 is a transcription factor exclusively expressed in the B lymphoid lineage from the earliest detectable precursor to the mature B cell stages [38,39], and controls the expression of CD19 [40]. Pax-5 was amplified in the population transfected with both the EGFP-transgene and the H-2K^k plasmid as well as in the control population transfected with the H-2K^k plasmid only. In turn, a band corresponding to the region of the IgH transgene was amplified, only in the population transfected with the EGFP-transgene (Fig. 3B). Taken together these results demonstrate that the small number of EGFP positive cells identified after spontaneous transgenesis includes cells of the B lineage. The fact that Pax-5 was also amplified in the control population reflects the presence of ~2% CD19⁺ cells.

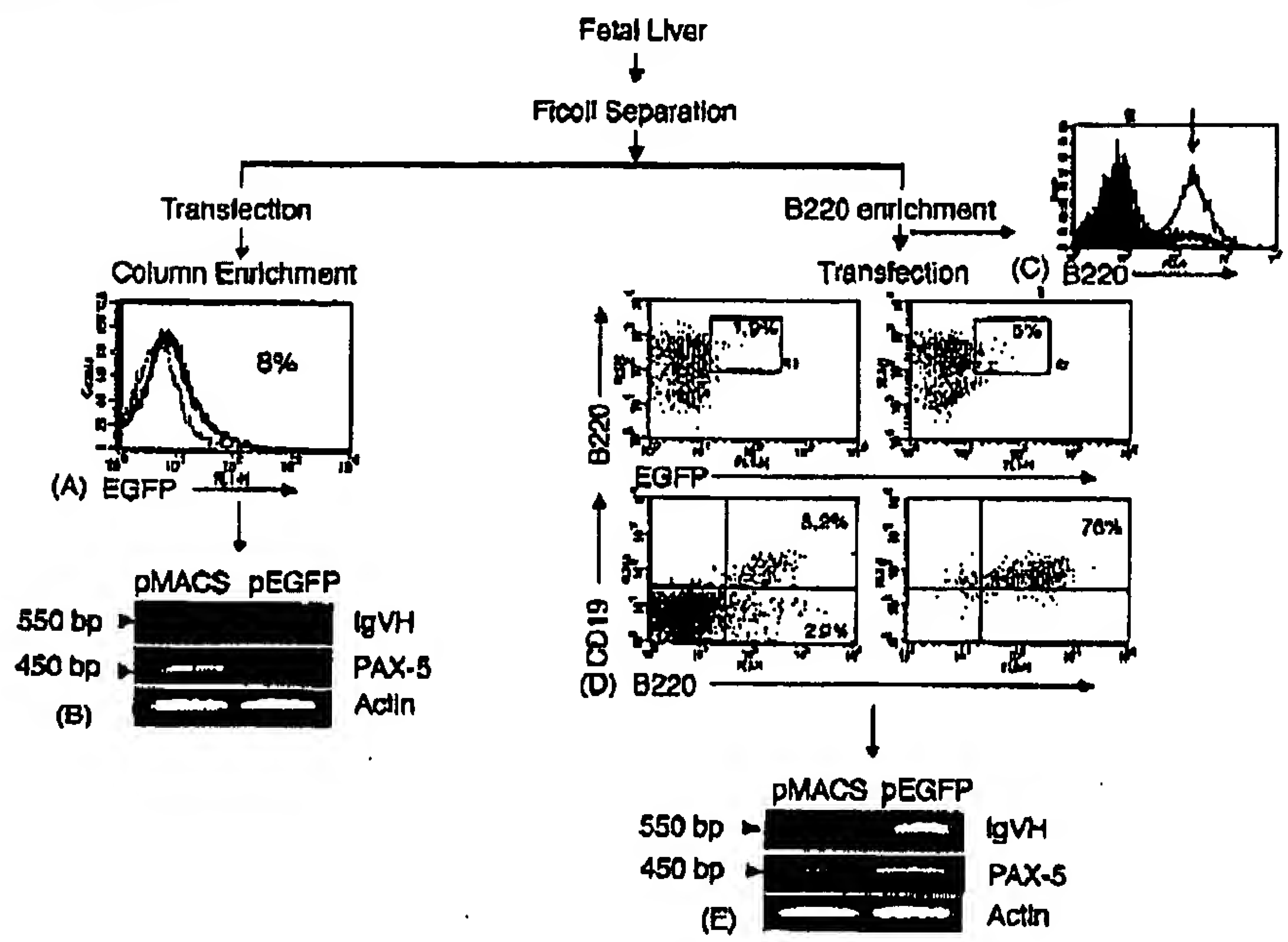

Fig. 3. Plasmid DNA targets B220$^+$ fetal liver cells. Fetal livers were collected 17 days post coitum and single cell suspensions were subject to Ficoll separation. (A) After spontaneous transgenesis and overnight incubation ~8% of pMACS-enriched cells express the green fluorescent protein. The dotted line shows cells transfected with only pMACS K^K DNA, and the solid line shows cells transfected with pMACS K^k DNA together with the EGFP-plasmid. Data are representative of four independent experiments. (B) Expression analysis by RT-PCR in pMACS-enriched cells. Lanes on the left refer to cells transfected with pMACS. Lane on the right refers to cells transfected with plasmid γ1NV^{2}NA^3 with the EGFP tag (pEGFP). Data are representative of four independent experiments. (C) Enrichment for B220$^+$ in Ficoll-separated liver cells. The filled hystogram represents non-stained cells, the solid black line B220$^+$ cells, and the dotted line B220$^+$-enriched cells. The arrow identifies the population that will be subject to spontaneous transgenesis. Data are representative of three independent experiments (D). B220$^+$-enriched cells incubated with pMACS K^K DNA (upper left panel). B220$^+$-enriched cells transfected with the EGFP-plasmid (upper right panel) (average transfection efficiency 2.5 ± 0.5), transfected cells double stained for CD19 and CD45R/B220 before enrichment and transfection (lower left panel), and enriched transfected cells double-stained for CD19 and CD45R/B220 (lower right panel). Data are representative of three independent experiments (E). Expression analysis by RT-PCR in B220$^+$-enriched cells. Lanes on the left refer to cells transfected with pMACS. Lanes on the right refer to cells transfected with plasmid γ1NV^{2}NA^3 with EGFP tag (pEGFP). Data are representative of three independent experiments.

Further proof for the involvement of fetal liver B cells in DNA uptake, transcription and expression, was sought in experiments in which B220$^+$ fetal liver cells were transfected and analyzed for EGFP positivity, and Pax-5 and IgH expression. To this end, fetal liver cells were first enriched (96%) on the basis of B220 expression (Fig. 3C). These cells were then subjected to spontaneous transgenesis. After 18 h, the cells were analyzed by FACS and RT-PCR. About 3% of the gated population expressed both EGFP and B220 (Fig. 3D, upper right panel). These cells were also B220/CD19 double positive (Fig. 3D, lower left) implying that this fetal liver population is susceptible to spontaneous transgenesis. This conclusion was corroborated by the amplification of Pax-5 and the IgH transgene from the RNA of B220$^+$ cells transfected either with the EGFP-transgene or the H-2K^k plasmid (Fig. 3E). Thus, even though the B220$^+$/CD19$^+$/EGFP$^+$ population is small in percentage, it is definitely a real population in light of the positivity for Pax-5 and the IgH transgene. Based on this phenotypic and RNA characterization, we conclude that the fetal liver contains committed B cell progenitors capable to uptake plasmid DNA, and express a transgene under the control of a B cell specific promoter. The discrepancy in the percentage in between total liver cell transfected and B220 enriched cells transfected indicates that B cells are not the only ones involved in this process.

Finally, to support the hypothesis that fetal liver B cells could serve as potential APC, further experiments were performed to document MHC Class II expression on the B220$^+$/CD19$^+$/Pax-5$^+$ cell population. To this end, liver cells from 16- or 17-day-old fetuses were prepared by Ficoll separation followed by B220 enrichment. These cells were then analyzed by FACS for MHC Class II (I-A^d) expression, immediately after preparation or after overnight incubation at 37 °C in RPMI medium. Both the B220$^+$ and the B220$^+$/CD19$^+$ cell populations comprise a small subpopulation of I-A^d positive lymphocytes (Fig. 4A and B). Notably, the number of CD19$^+$/I-A^d positive cells in fetal liver increases markedly on day 17 and so did the mean fluorescence intensity for I-A^d after overnight incubation. Expression of the MHC Class II molecule in the B220$^+$/CD19$^+$/I-A^d pos-

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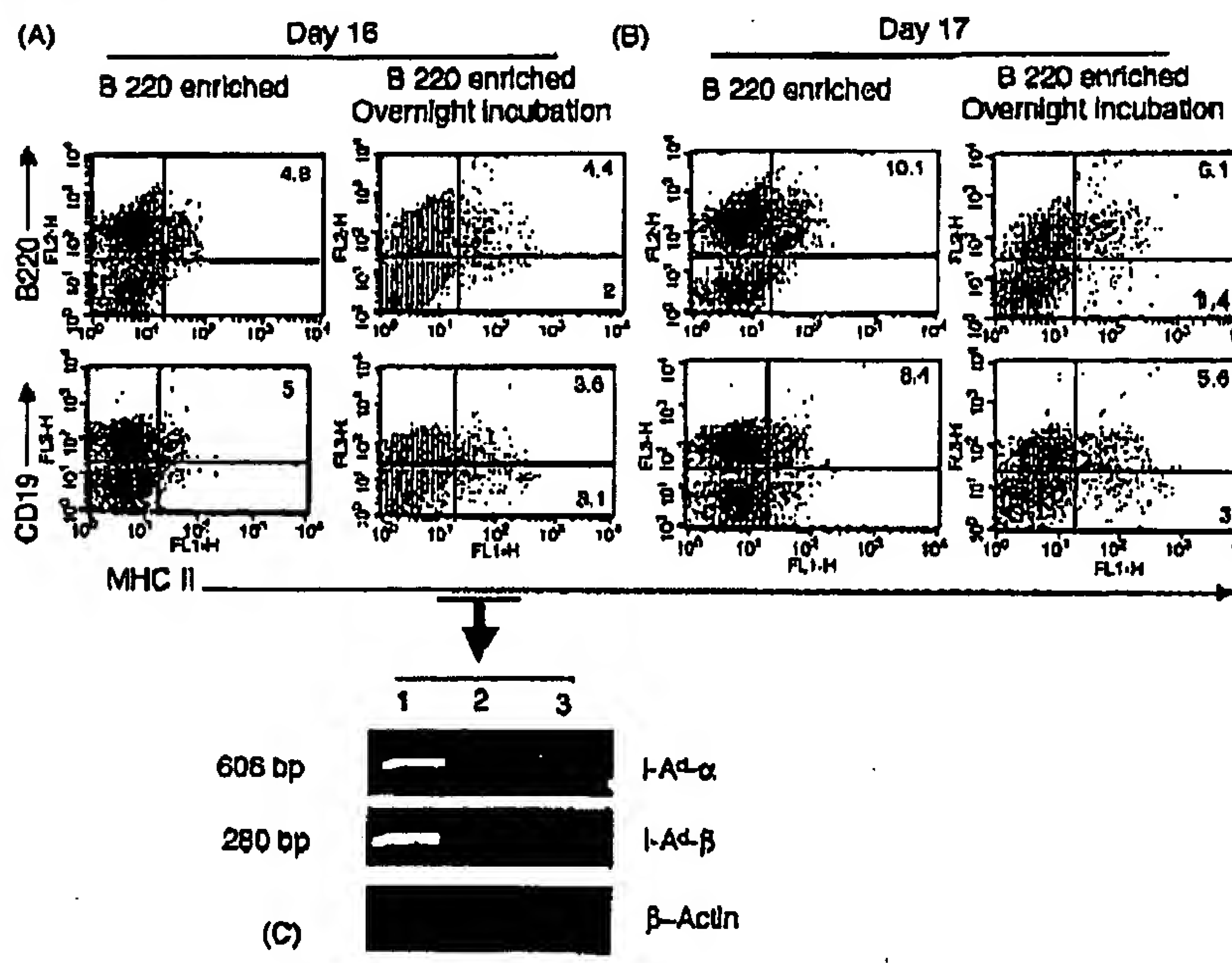

Fig. 4. MHC Class II expression on fetal liver B220+ cells. Fetal liver cells were collected either 16 (A) or 17 (B) days post coitum. Ficoll separated B220+ enriched cells were stained for CD19 and I-A^d expression. In A and B, the left panels refer to staining immediately after B220 enrichment, and right panels refer to staining performed after overnight incubation in RPMI 10% FCS. Cells after overnight incubation were RNA extracted in both instances and analyzed for I-A^d α and β expression, β actin and Pax-5 (not shown). Lane 1 correspond to splenocytes of adult Balb/c mice as control, lane 2 to fetal liver cell after Ficoll separation, and lane 3 to B220+ enriched cells after overnight incubation. Data shown are representative of two experiments.

itive subpopulation in fetal liver was confirmed by RT-PCR of the α and β chains of the I-A^d molecule. Fig. 4C shows a band corresponding to the α and β chains of I-A^d amplified from the RNA of B220+-enriched population of a day 16 fetal liver. Interestingly, fetal liver cells after Ficoll separation and overnight incubation were negative implying that the enrichment step is necessary to visualize MHC Class II+ cells in fetal liver. The presence of a band corresponding to Pax-5 (not shown) confirmed that these amplification products were originated from B lineage cells. These data prove, therefore, the fetal liver comprises a small population of B220+/CD19+ B cells, which express MHC Class II molecules.

3. Discussion

In this paper, we show that T cell immunity of the Th1 type can be programmed in utero by manipulating the parameters of the immune response in the fetal environment, yielding effector CD4 T cells 7 and 15 days after birth and a memory T cell response. We also show that the antigen can be synthesized in fetal liver B lymphocytes, and we hypothesize a role for these cells for T cell priming. We conclude that DNA vaccination in utero can lead to efficient priming of specific T cell responses and that under appropriate conditions the fetal immune system can be programmed toward immunity.

Prenatal immunization yielded a Th1 response in the vast majority of pups from responder litters by the end of the first week of life. A Th1 response this early in ontogeny contrasts with the known preferential Th2 polarization of neonatal responses after vaccination [13]. Did T cell priming occur in utero or was the immune response already present at birth? We reasoned that if priming was a postnatal event we would be able to induce T cell priming by immunizing soon after birth. However, no response was found in mice inoculated intra-peritoneally with plasmid DNA 1 (0/12) or 7 (0/8) days after birth, and no memory response followed a booster immunization on day 45 (data not shown). These findings suggest that either neonatal B cells and their precursors are no longer susceptible to DNA uptake locally or that at the time of birth B lymphocytes home to the spleen and bone marrow where they participate in organ remodeling [41,42]. Compared with the refractoriness of T cell priming following postnatal immunization, these results indicate that the late stage fetal immune system offers a window of opportunity for T cell priming and the initiation of T cell responses.

Although it was not possible to directly demonstrate in vivo targeting of functional fetal liver B lymphocytes, we hypothesize that this event is the likely substrate for expression of the transgene in utero. This would represent the first step in the initiation of T cell priming. For instance, it is known that adult B cells process and present to CD4 T lympho-

cytes endogenous Ig peptides in vitro [43] and in vivo [44]. Similar function can be postulated for fetal liver B lymphocytes. However, in as much as the ex vivo studies showed expression of plasmid DNA in CD19/B220 double positive B lymphocytes, we cannot rule out the possibility that fetal cells other than B cells may uptake and express the transgene if at this stage of ontogeny the B cell promoter is not restricted in its activity. The possibility that fetal dendritic cells may be responsible for T cell priming is weakened by the fact that these cells constitutively express indoleamine 2,3-dioxigenase (IDO) [45,46], an enzyme involved in the catabolism of tryptophan, whose up-regulation in dendritic cells is promoted by IFN-γ [47]. Since, dendritic cells expressing IDO suppress T cell function and cause apoptosis of T cells [48–50], their possible role in T cell priming during fetal life is highly unlikely. In fact, this is consistent with the view that IDO represents a safekeeping, tolerogenic mechanism [51] against untimely rejection of the fetus by the mother [52]. A second possibility is that dendritic cells at the maternal fetal interface express high level of HLA G molecules, which induce suppressor/regulatory CD4 T cells [53]. One cannot rule out that transfected liver cells could be metastable cells deriving from the fusion with lymphopoietic cells, as demonstrated recently for liver cells and bone marrow stem cells [54], or vice versa.

The results presented here could not have been predicted on the basis of reports showing that fetal liver pre-B cells do not express MHC Class II molecules. Here we show that a small fraction of B220$^+$/CD19$^+$/Pax-5$^+$ fetal liver B lymphocytes are reactive with monoclonal antibody against I-A^d. A reason for this discrepancy could be simply due to the different isolation procedure used here, that is the separation of fetal liver cells by Ficoll centrifugation to enrich in lymphoid cells followed by isolation of B220$^+$ cells to further enrich for B lineage cells. Since, CD19$^+$ cells in the liver begin to express surface IgM by day 15 [55], and surface IgM expression coincides with MHC Class II expression [56], the results presented here are consistent with the view that fetal B lineage progenitors may not be an entirely different lineage from those in the adult bone marrow [55], as previously proposed [37,56]. Since, during fetal/neonatal development B cell progenitors migrate from the liver to the bone marrow and the spleen [42], the functional study presented here strengthens the notion that fetal liver B cell progenitors are a diverse population from which originate the founders for the early B cell lymphopoiesis in the bone marrow and the spleen postnatally. Two arguments make us believe that in our experiments the stage for inducing immunity was likely set within the fetal liver. One is that in the mouse the migration of lymphoid cells from the liver to the bone marrow and the spleen is completed few days after birth [42]. The other is that we failed to induce immunity by postnatal immunization. Then a plausible scenario is that the process of fetal immunization evolves from the liver following the dynamics of lymphopoiesis and lymphoid organogenesis from fetal to neonatal life, respectively.

The success of fetal immunization demonstrated here is owed, in our opinion, to the preset characteristics of the immunization process. We speculate that the characteristics of fetal immunization shown here reflect a system program where fetal transgenic B cells, while part of the dynamics of organ lymphopoiesis, proceed to express antigen (the transgenic product), and where the characteristics of the immunization process mimic infection with a non-cytopathic virus. These considerations on immune dynamics also provide an opportunity for reinterpreting the results of the study of Sarzotti et al. [16] where induction of cytotoxic T lymphocytes was obtained by neonatal infection with a very low dose of a B lymphotropic oncovirus. However, whether or not a mechanism similar to the one described here could be operative in fetal life under physiological circumstances is presently unknown.

Neonatal immunization is required to afford protection against diseases caused by intracellular pathogens, such as respiratory syncytial virus, herpes simplex viruses, cytomegalovirus, HIV and malaria. Evidence is beginning to accumulate that the fetal immune system may be sensitized to antigen [19] and specific CTL that may originate from fetal sensitization contain viral load after birth [57]. Thus, fetal priming and perinatal boosting with a vaccine strategy both tailored to overcome tolerance induction while maintaining a safety profile, could allow better control of infection diseases early in life. The immunization shown here met key immunological criteria such as immunogenicity and induction of mainly Th1 responses. Antibodies specific for γ1NV^{2}NA^3 were also detected 2 weeks after birth in 16 out of 17 immunized mice with a titer ($\log_{10}$) ranging between 2–3.2 versus <2.6 in mice injected with control DNA. The immunization approach shown here also met initial safety profile in mice in that the transgene was inconsistently found in lymphoid tissues after birth but not after day 7, was never found in non-lymphoid organs, and no vertical transmission of the transgene was observed when females and males injected in utero were mated in adult life (our unpublished data). Further studies will be needed for any adaptation of the approach shown here to humans.

In summary, the present data demonstrate that by manipulating the parameters of the immune response to conform with the dynamics of the fetal environment, immunity and not tolerance is attained. Because the characteristics of the T cell immunity are that of a Th1 response, and this is needed to control infection caused by intracellular pathogens, the system program shown here suggests the principle for successful prophylactic or therapeutic vaccinations against infectious agents during pregnancy. Since, it is now clear that CD4 T cell immunity is necessary at the time of priming to induce memory CD8 T cells [58–60] and heightened protective memory CD8 T cell responses [61], the results shown here demonstrate that, contrary to a commonly held paradigm, effective adaptive T cell responses can be induced by immunization in utero. We propose that the fetal immune system can be tilted to immunity or tolerance depending on the antigen-presenting

cell, the amount and the source of antigen (exogenous versus endogenous), and the relation of these factors with the timing of the development of fetal lymphoid organs.

4. Materials and methods

4.1. Mice

Eight-to ten-week-old Balb/c (H-2^d) mice were purchased from The Jackson Laboratory, kept in the animal facility of the University of California, San Diego, and handled according to institutional and federal regulations. To generate pregnancies, cages were set with two females and one male. After 3 days, mice were separated and this time was considered the first day of gestation. Time of gestation was additionally determined by a combination of vaginal plug detection and/or expert eye inspection at the time of injection. In each instance, delivery occurred two and half-three days after the in utero injection.

4.2. Plasmid DNA and synthetic peptides

Plasmid γ1NV^{2}NA^3 is as described previously [23]. Plasmid pSVneo served as a control. Synthetic peptide NANP NVDPNANP(-NVDP-) and its control NANPNANPNANP $(NANP)_3$ were synthesized at the peptide chemistry facility of the California Institute of Technology (Pasadena, CA).

4.3. Immunization of fetuses in utero

On day 16 of gestation, pregnant BALB/c were anesthetized by mask anesthesia using 2% isoflurane. Midline laparotomy was performed and each horn of the gravid uterus was exposed. Fetuses were injected trans-utero into the peritoneal cavity with 5 μg of plasmid γ1NV^{2}NA^3 in 5 μl of sterile PBS using a Microliter™ no. 701 Hamilton syringe (Hamilton, Reno, NV). Following injection, the utero was returned to the abdominal cavity, and the abdominal wall was sutured with 5-0 Dexon S (USS DG sutures) [62]. Control fetuses were injected with plasmid pSVneo, or simply PBS. Pups were sacrificed 3, 7 or 14 days after birth. For studies on immunological memory, f mice were given a booster immunization by subcutaneous injection (50 μg/mouse) of affinity purified transfectoma antibody γ1NV^{2}NA^3 [23] emulsified in incomplete Freund's adjuvant (IFA) on day 45 after birth and sacrificed 4 days later.

4.4. T cell assays

Proliferative responses were assessed by ^{3}[H]-Thymidine incorporation on spleen lymphocytes as described [23]. Results are expressed either as counts per minute (cpm) or stimulation index calculated as ratio of (cpm of cell cultured in the presence of synthetic peptide)/(cpm of cell cultured in the absence of peptide). ConA stimulated cultures were used as positive indicator of proliferation. For the purpose of cytokines detection, culture supernatants were harvested at 40 h and stored at −20 °C. The supernatants from three separate triplicate cultures were pooled for each mouse. IL-2, IL-4 and IFN-γ were measured in the same 40-h culture supernatants by ELISA using the OptEIa Elisa kit (Pharmingen, La Jolla, CA). Standard curves were constructed using purified IL-2, IL-4, and IFN-γ. Tests were done in duplicate.

4.5. Preparation of fetal liver cells and performance of spontaneous transgenesis

Fetal livers were collected from fetuses at 17 days of gestation and gently homogenized by pipetting. Cell suspensions were then filtered through a cell strainer (70 mM, Falcon, BD, La Jolla, CA) to eliminate debris. Lymphoid cells were then separated by Ficoll centrifugation (Lympholyte®-M, Cedarlane, Hornby, Ont.). Separated cells were washed three times in phosphate buffer saline (PBS) and re-suspended 4×10^6 in 200 μl of PBS without Ca^{2+} and Mg^{2+} and incubated for 60 min at 37 °C in a CO_2 atmosphere with 25 μg of plasmid-EGFP together with 5 μg of PMACS K^k plasmid (Miltenyi Biotec, Auburn, CA). PMACS K^k codes for a truncated mouse H-2 K^k molecule as a selectable cell surface marker. These cells were then washed and incubated overnight in culture medium at 37 °C in 5% CO_2 atmosphere. The cells were harvested and re-suspended in 320 μl PBS/0.5% BSA/5 mM EDTA (PBE) containing 80 μl of MACSselect K^k microbeads. The suspension was incubated for 30 min at 4°C. Transgenic and magnetically labeled cells were sorted by positive selection on a MS+/RS+ column mounted on the magnetic field of a MACS separator. Negative cells were collected in the flow-through. After washing, the column was removed from the separator and transgenic cells were flushed out with PBE using a plunger.

4.6. Flow cytometry

After spontaneous transgenesis, cells were analyzed by FACS according to the schema shown in Fig. 3. Cells transfected with the magnetic plasmid only served as negative control. A gate in the forward versus side scatter map was set to exclude debris and dead cells. After washing, cells were resuspended in 300 μl of PBS and analyzed on a FACsCalibur (Beckton & Dickinson, CA). The phenotype of the transfected cells was determined on magnetically separated cells after overnight culture. Cells were washed with PBS containing 0.5% BSA and 0.05% NaN_3, and stained with a perCP-cy5.5 conjugated monoclonal antibody to murine CD19 and one of the following PE-conjugated monoclonal antibodies to murine CD45R/B220, I-A^d or H2-K^d (Pharmingen). Cells were analyzed on a FACsCalibur.

4.7. B220 enrichment

Ficoll-separated pooled fetal liver cells were washed in PBE, divided in aliquots of 10^7 cells and incubated with a

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PE-conjugated monoclonal antibody to murine CD45R/B220 (Pharmingen) for 10 min at 4 °C. Cells were then washed with PBE and 20 μl of anti-PE beads were added to the cell suspension. After incubation at 4 °C for 20 min, magnetically labeled cells were separated as indicated above. After enrichment, $B220^+$ cells were analyzed by FACS, then subject to spontaneous transfection as described above. After overnight culture in RPMI 10% FCS, the cells were collected, analyzed by FACS and the RNA extracted.

4.8. RT-PCR

Total RNA was extracted from 10^5 transfected, non-transfected or B220-enriched fetal liver cells. Cells were washed three times in PBS to eliminate contaminant DNA. RNA extraction was performed with RNeasy Mini kit (Quiagen, Valencia, CA) with the support of 50 ng of yeast tRNA (Invitrogen, San Diego, CA). DNAse digestion (Rnase free-Dnase set, Qiagen) was performed on the column during RNA purification. cDNA was prepared using polyT synthesis with Omniscript RT (Qiagen) for 1 h at 37 °C. PCR was performed with a total of four sets of primers. Gammafwd (5′-TCAAGGACTACTTCCCCGAACC-3′) and gammafwd2 (5′TACTCCTTGCCATTCAGCCAGTCC-3′) were used to amplify for 30 cycles a sequence of the human IgH gene. The nested reaction used Negammal (5′-GCAGCTTGGGCACCCAGACCTACA-3′) and Negamma2 (5′-CCGCGGCTTTGTCTTGGCATTATG-3′) for a subsequent 30 cycles, amplifying a band of ~550 bp. The annealing temperature for both of primers was 64 °C. Pax-5 up (5′-CTACAGGCTCCGTGACGCAG-3′) and Pax-5 down (5′-GTCTCGGCCTGTGACAATAGG-3′) as described [63] were used twice for 30 cycles on the same product with annealing temperature at 61 °C amplifying a band of ~450 bp. β-Actin was used as a control of extraction. Samples were subsequently analyzed on a 1.5% agarose gel. For the amplification of MHC Class II I-A^d two sets of primers have been used. The first amplifying the α chain of the MHC, the second the β chain. Primers were design on different exons allowing to distinguish RNA bands from contaminants DNA bands. Sequence of primers was the following I-A-α up 5′-ACTGTCTGGAGGCTTCCTGAGTTTG-3′, I-A-α down 5′-AGAGGGACACACACCTTCCTTTCC-3′ amplifying a band of 606 bp and I-A-β up 5′-CCACCACAACACTCTGGTCTGTTC-3′I-A-β down 5′-TGCCGCTCAACATCTTGCTCCG-3′ amplifying a band of 280 bp. PCR conditions were as described [37].

Acknowledgments

This work was supported in part by NIH grant RO1CA92119. M.R. was recipient of a fellowship from the Giannini Family Foundation. We thank Jim Feramisco, Javier Hernandez, Hyam Leffert, Ramon Pinon, Linda Sherman for constructive discussions during the development of this project; and Dennis Young, April Simora and Tom Kaido for help and advice.

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